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A Novel High-Resolution Melt PCR Assay Discriminates *Anaplasma phagocytophilum* and "*Candidatus* Neoehrlichia mikurensis"

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"*Candidatus* Neoehrlichia mikurensis" (*Anaplasmataceae*) is an emerging pathogen transmitted by *Ixodes* ticks. Conventional PCR and the newly developed high-resolution melt PCR were used to detect and discriminate "*Candidatus* Neoehrlichia mikurensis" and *Anaplasma phagocytophilum*. Both bacterial species were frequently found in *Ixodes ricinus* and *Ixodes hexagonus* but virtually absent from *Dermacentor reticulatus*. In rodents, "*Candidatus* N. mikurensis" was significantly more prevalent than *A. phagocytophilum*, whereas in cats, only *A. phagocytophilum* was found.

Pathogenic *Anaplasmataceae* of the genera *Anaplasma*, *Ehrlichia*, and *Neoehrlichia* are transmitted by ixodidae. *Anaplasma phagocytophilum*, the agent of human, equine, canine, and feline granulocytic anaplasmosis (GA) and tick-borne fever of ruminants, occurs throughout central and northern Europe (1). *Anaplasma phagocytophilum* genotypes in North America and Europe apparently differ in host tropisms and clinical symptoms, with ruminant tick-borne fever and equine GA found predominantly in Europe and human GA predominantly in North America (1). Although human GA is often asymptomatic, 7% of diagnosed clinical cases require intensive medical care and 0.6% of the cases are fatal (2). *Anaplasma phagocytophilum* is transmitted by *Ixodes* species. In Eurasia, vector competence has not been experimentally established for any tick species, but the bacterium was detected in many different *Ixodes* species (1), including *Ixodes ricinus* (3, 4) and *Ixodes hexagonus* (1, 5).

Recently, "*Candidatus* Neoehrlichia mikurensis" was identified as a new member of the *Anaplasmataceae*. This emerging pathogen is also transmitted by *Ixodes* ticks and can cause severe disease in humans (6–8) and dogs (9). It was first identified in *Ixodes ovatus* and *Rattus norvegicus* in Japan (10). Bacterial DNA was detected in liver, heart, and spleen tissue, and intracellular bacteria were found in splenic endothelial cells. In two human cases, the pathogen occurred in granulocytes (11). Previously, very closely related 16S rRNA gene sequences were reported as the Schotti variant of *Ehrlichia*-like bacteria in the Netherlands (12) and *Ehrlichia* sp. "*Rattus* Strain" in China (13). "*Candidatus* Neoehrlichia mikurensis" appears to be one of the most prevalent pathogens in *I. ricinus* in Europe (14–17), suggesting the possibility that many subclinical human infections occur. Therefore, the aim was to develop a real-time high-resolution melt PCR (HRM-PCR) assay discriminating *A. phagocytophilum* and "*Candidatus* Neoehrlichia mikurensis."

Six rodent species were trapped in 2010 and 2011 at four different sites in Berlin (Table 1). Spleen samples were collected during necropsy from cats from animal shelters in Berlin (2006 to 2008). Host-associated, nonengorged, or partially engorged *I. ricinus* and *I. hexagonus* ticks were collected from dogs in Berlin and Brandenburg by their owners and sent to the Small Animal Clinic, Freie Universität Berlin, Berlin, Germany. Questing *Dermacentor reticulatus* ticks were collected by flagging in recreational areas in Berlin and Brandenburg. Additionally, adult male ticks from several batches originating from 12 different females of our *I. ricinus* breeding colony were tested. For feeding the various stages of *I. ricinus* ticks, laboratory-bred gerbils and rabbits were used exclusively and only once. Dog blood samples on Whatmann FTA cards were obtained from veterinarians for diagnostic purposes.

Following DNA isolation, a PCR amplifying a 257-bp fragment of the 16S rRNA gene of most *Anaplasma*/*Ehrlichia* species (18, 19) was modified by introducing an additional wobble position to include further species. PCR conditions are given in Table S1 in the supplemental material. As controls, samples containing 20 and 1,000 copies of cloned PCR products were run in parallel. Melting curves were analyzed using Bio-Rad Precision Melt Analysis software. For species identification, the melt-curve shape parameter and melting temperature threshold were set to 50 and 0.25°C, respectively. Samples were considered positive when they clustered with one of the plasmid standards irrespective of quantification cycle (c_q) values. Prevalence differences were calculated with the mid-P exact test (20).

All PCR products obtained by amplifying anaplasmataceae-specific 16S rRNA gene fragments in rodent, feline, *D. reticulatus*, and 88.9% of the *I. ricinus* samples were examined by sequencing. The remaining 11.1% of *I. ricinus* and all *I. hexagonus* samples were analyzed only by HRM. Either *A. phagocytophilum* or "*Candidatus* Neoehrlichia mikurensis" was identified. In one *Apodemus agrarius* sample, a coinfection was revealed by multiple peaks

Received 13 February 2013 **Returned for modification** 12 March 2013
Accepted 4 April 2013
Published ahead of print 10 April 2013
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† Deceased 5 September 2011.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.00284-13.

doi:10.1128/JCM.00284-13

TABLE 1 Prevalence of *Anaplasma phagocytophilum* and "*Candidatus* Neoehrlichia mikurensis" in different host and vector species examined by conventional and high-resolution melt PCR[a]

Species	*A. phagocytophilum*		"*Candidatus* N. mikurensis"		*P* value[b]
	Prop.	Prev. (95% CI)	Prop.	Prev. (95% CI)	
Rodents					
Myodes glareolus	0/56	0 (0–7.7)	16/56	28.6 (18.3–41.6)	<0.001
Microtus arvalis	0/11	0 (0–30.0)	4/11	36.4 (15.9–64.8)	0.045
Microtus agrestis	0/2	0 (0–71.0)	2/2	100 (29.0–100)	0.167
Apodemus flavicollis	4/82	4.9 (1.5–11.4)	10/82	12.2 (6.6–21.2)	0.167
Apodemus agrarius	1/78	1.3 (0.1–7.6)	4/78	5.2 (1.6–12.9)	0.213
Apodemus sylvaticus	2/25	8.0 (1.1–26.1)	0/25	0 (0–15.8)	0.245
All rodents	7/254	2.8 (1.2–5.7)	36/254	14.2 (10.4–19.0)	<0.001
Cats	6/141	4.3 (1.8–9.7)	0/141	0 (0–3.2)	0.015
D. reticulatus[c]	1/1,237	0.08 (0.004–0.5)	1/1,237	0.08 (0.004–0.5)	1
I. hexagonus[d]	5/151	3.3 (1.2–7.2)	10/151	6.6 (3.5–11.9)	0.199
I. ricinus[d]	49/773	6.3 (4.8–8.3)	32/773	4.1 (2.9–5.8)	0.053
I. ricinus (breeding colony)	4/49	8.2 (2.7–19.7)	0/49	0 (0–8.6)	0.058[e]

[a] Prop., proportion (number of animals infected/total number of animals); Prev., prevalence with 95% confidence interval (CI).
[b] Significant difference between prevalence of *A. phagocytophilum* and *Candidatus* N. mikurensis calculated using the mid-P exact test.
[c] Questing adult ticks.
[d] Dog-associated ticks, most of them partially engorged.
[e] When the uncorrected chi-square test or the Mantel-Haenszel chi-square test is used, this *P* value is < 0.05.

in the electropherogram (see Fig. S1 in the supplemental material). A few cat samples were positive for *A. phagocytophilum*, but none were positive for "*Candidatus* Neoehrlichia mikurensis."

Since no unspecific PCR products were detected in ticks, a HRM assay was developed for rapid species discrimination. The method reliably provided specific results for rodent and tick samples and detected as few as 20 copies (minimum of copy numbers tested), as determined by analysis of cloned PCR products. The agreement between HRM-PCR and conventional PCR was 100% ($n = 42$). Figure 1 shows representative HRM-PCR results for a subset of *A. phagocytophilum*- and "*Candidatus* Neoehrlichia mikurensis"-positive samples. A clear distinction between the melting curves for both bacteria is evident. One doubly positive sample unexpectedly but reproducibly denatured at a much lower melting temperature and did not provide two peaks representing the different species (the experiment was repeated four times). In order to prove that the method is also suitable to discriminate other relevant *Anaplasmataceae* species, two blood samples, previously tested positive for *Ehrlichia canis* by conventional PCR and sequencing, were analyzed by HRM-PCR. For all three species, cloned PCR products were again used as the templates and run in parallel with the samples as positive controls. Both blood samples could unequivocally be identified as *E. canis* compared with *A. phagocytophilum* and "*Candidatus* Neoehrlichia mikurensis" (see Fig. S2 in the supplemental material).

In rodents, the prevalence of "*Candidatus* Neoehrlichia mikurensis" was about 5-fold higher than that of *A. phagocytophilum* (Table 1). In individual rodent species, significant differences in the prevalences of the two pathogens were found only for *Myodes glareolus* and *Microtus arvalis.* In these species, no *A. phagocytophilum* was detected, although *Myodes* spp., including *M. glareolus*, were previously reported to be infected with *A. phagocytophilum* (21). Although *Apodemus sylvaticus* mice in the Netherlands were shown to be frequently infected with "*Candidatus* Neoehrlichia mikurensis" (15), this pathogen was not detected in these mice in this study ($n = 25$).

Only one *D. reticulatus* sample was positive for each pathogen, further suggesting that this tick has no epidemiological relevance in the transmission of *Anaplasmataceae* (22). Both *Ixodes* species were frequently infected with one of the two bacterial species, but no coinfections were observed. The prevalences of these pathogens did not differ significantly in dog-associated ticks. Ticks from our *I. ricinus* breeding colony were initially tested by conventional PCR. For positive samples, the pathogen species was identified by HRM-PCR. Results (Table 1) confirmed vertical transmission of *A. phagocytophilum* (1) but not of "*Candidatus* Neoehrlichia mikurensis" (15).

A rapid, sensitive, and reliable diagnostic tool was developed that allows clear differentiation of the two members of *Anaplasmataceae* prevalent in central and northern Europe. Similar to a recently published probe-based real-time multiplex PCR to detect *A. phagocytophilum* and "*Candidatus* Neoehrlichia mikurensis" (15), the method described here is capable of detecting and discriminating both species. HRM is less expensive than probe-based detection but is in principle also able to additionally detect and differentiate other species of the *Anaplasmataceae*, (e.g., *E. canis*). Other *Anaplasmataceae*, in particular, *E. canis* (23) but presumably also *Anaplasma platys*, are frequently imported into northern European countries by introducing infected dogs. However, these bacteria are thus far not known to be transmitted in an autochthonous manner by any endemic tick species (1); therefore, establishment of stable transmission foci is currently unlikely. For differential diagnoses in dogs, they might nevertheless be relevant.

The ability of different rodents or other mammals to serve as reservoir hosts for "*Candidatus* Neoehrlichia mikurensis" has not yet been examined experimentally and needs to be determined. Other wildlife animals (e.g., roe/red deer, mouflon, wild boar, domestic ruminants, and birds) should also be considered. The prevalences of "*Candidatus* Neoehrlichia mikurensis" in different rodent species reported here and in the Netherlands (15) differ greatly, suggesting that this pathogen is able to infect many different rodents. Thus, epidemiologically important reservoir hosts

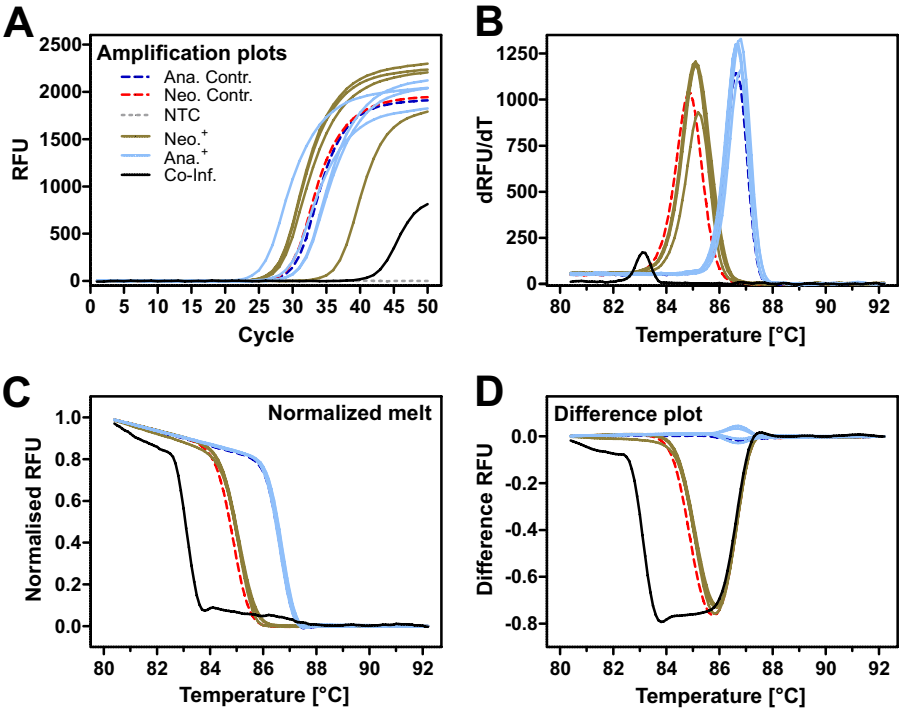

FIG 1 High-resolution melt PCR for discrimination of *A. phagocytophilum* and "*Candidatus* Neoehrlichia mikurensis." (A) Amplification plots of 8 different tick samples positive for *A. phagocytophilum* (Ana.$^{+}$) or "*Candidatus* Neoehrlichia mikurensis" (Neo.$^{+}$) and a doubly positive spleen sample from *A. agrarius* (Co-Inf.), positive controls for both pathogens (Ana. Contr., Neo. Contr.), and a no-template control (NTC), showing relative fluorescence units (RFU) versus cycle numbers. (B) First derivative of melting curves against the temperature (*dF*/*dT*) of the same data set whose results are presented in panel A. (C) Normalized melting curves of the same data set. (D) Difference plots with the mean value for *A. phagocytophilum*-positive samples subtracted from all individual samples. In the Precision Melt Analysis software, the melt curve shape parameter of 50 was used and the melting temperature threshold was set to 0.25°C. The software clearly allocated the melting curves to three clusters corresponding to *A. phagocytophilum*, "*Candidatus* Neoehrlichia mikurensis," and the double infection. Early melting of the amplicon obtained from the coinfected *A. agrarius* was probably due to formation of heteroduplex DNA during the annealing step in late PCR cycles.

probably need to be identified in each ecosystem. The absence of "*Candidatus* Neoehrlichia mikurensis" in feline samples suggests that this pathogen may not establish infections in cats or may establish such infections only rarely, indicating a minor or no role for this companion animal in the transmission cycle.

No hints of transovarial transmission of "*Candidatus* Neoehrlichia mikurensis" were found in flagged *I. ricinus* larvae (15). *I. ricinus* ticks from our breeding colony were obtained from Berlin and Brandenburg, and it can be expected that the prevalence of "*Candidatus* Neoehrlichia mikurensis" and *A. phagocytophilum* in these adult ticks was comparable to the prevalence in the *I. ricinus* ticks investigated in this and other studies (15, 17). After 2 to 4 generations of ticks were fed solely on laboratory animals, "*Candidatus* Neoehrlichia mikurensis" was not detectable in any of the examined batches (only adult males were tested) from different time points. In contrast, *A. phagocytophilum* was detected in the same ticks from this breeding colony, confirming transovarial transmission. The prevalence of "*Candidatus* Neoehrlichia mikurensis" in questing ticks is at least comparable to (this study) or significantly higher than (5) the prevalence of *A. phagocytophilum*. The results obtained for adult ticks of the breeding colony suggest that transovarial transmission is, at least, much rarer for "*Candidatus* Neoehrlichia mikurensis" than for *A. phagocytophilum*, further corroborating results of Jahfari et al. (15).

Tick-borne diseases are considered to impose particular health risks for people in rural areas. Obviously, "*Candidatus* Neoehrlichia mikurensis" is highly prevalent in ticks and rodents throughout the Berlin metropolitan area, including densely populated districts. This suggests that people and their companion animals in large cities have a considerable risk of encountering tick-borne pathogens. Investigation of *I. ricinus* collected from humans demonstrated a high prevalence of "*Candidatus* Neoehrlichia mikurensis" but the absence of any signs of vector-borne disease in humans (17). Further surveys, preferably also involving specific serology together with clinical data, are required to estimate the current health risk associated with "*Candidatus* Neoehrlichia mikurensis." The described HRM method will allow epidemiological surveys at significantly lower costs than studies based on species identification using sequencing or multiplex real-time analysis performed with hydrolysis probes. Additionally, it will allow rapid screening of potential reservoir hosts to identify those species whose reservoir competence should be characterized experimentally.

ACKNOWLEDGMENT

We thank Julia Blümke for her substantial support in capturing rodents.

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